ANTIHYPOXIC PROPERTIES OF THE BRAIN-DERIVED NEUROTROPHIC FACTOR IN THE MODELING OF HYPOXIA IN DISSOCIATED HIPPOCAMPAL CULTURES

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The aim of the investigation was to study the effect of brain-derived neurotrophic factor (BDNF) on the survival and main parameters of bioelectrical activity of neuronal network in primary hippocampal culture in short-term hypoxia and within 7 days of posthypoxic period *in vitro*.

Materials and Methods. The investigation was performed using dissociated hippocampal cells taken from embryonic CBA mice (E18) and plated on multielectrode arrays and cultured for 33 days. Modeling of hypoxia was performed on 33 day of culture development *in vitro* (DIV) by replacing the normoxic culture medium by a medium with low oxygen for 10 minutes. BDNF (1 ng/ml) was added 20 minutes before hypoxic damage.

Results. 10-minute hypoxia with an oxygen content in the medium of 0.37 ml/l after a short period of normalization of activity after reoxyganation was stated to lead to irreversible inhibition of the spontaneous bioelectrical activity of dissociated hippocampal cultures in remote posthypoxic period accompanied by the increase of the number of dead cells by 4.2 times. The addition of 1 ng/ml BDNF into the culture medium 20 minutes before changing normoxic medium for medium with low oxygen saved bioelectrical activity both in the process of hypoxia itself and in posthypoxic period, and increased cell survival.

Conclusion. Preventive application of BDNF reduces the negative consequences of normobaric hypoxia that enables to consider the neurotrophic factor as the substance having not only neuroprotective, but also antihypoxic properties.

Key words: brain-derived neurotrophic factor (BDNF), hypoxia, neuroprotection, dissociated hippocampal culture, multielectrode arrays.

Hypoxia is one of the main factors of brain cell damage in ischemia and some other pathologies. Due to alteration in oxygen supply there are significant changes of synaptic transmission processes related to cell death and cerebral neuronal network damage [1]. Therefore, in modern neurobiology and medicine the topical question is that of the search of substances able to protect brain cells from damaging effect of hypoxia. Among chemicals able to control cell metabolic rate under hypoxia, a group of regulatory proteins and peptides is distinguished. One of the representatives of this group is brain-derived neurotrophic factor (BDNF) that participates not only in neuron differentiation and formation of synaptic contacts in neurogenesis but can also be an active corrector of mature neurons metabolism [2–7]. However, its role in the oxidative process regulation in mature brain is not adequately investigated.

The aim of the investigation was to study the effect of brain-derived neurotrophic factor on the survival and main

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parameters of bioelectrical activity of neuronal network in primary hippocampal culture in short-term hypoxia and within 7 days of post-hypoxic period *in vitro*.

Materials and Methods.

Primary dissociated hippocampal cells culture. The research was carried out on hippocampal cells dissociated from 18-day embryos of CBA mice. The basic rules of experimental animals management and care met those represented in the Order of the Ministry of Health and Social Development of Russian Federation No. 708н, dated August 23, 2010, "Approval of laboratory practice rules". When carrying out investigations, ethical principles were kept inviolate according to European Convention for the protection of vertebrata used for experimental and other scientific purposes (the convention was passed in Strasburg, 18.03.1986, and adopted in Strasburg, 15.06.2006).

The cells were dissociated by treating hippocampal tissues by 0.25% trypsin (Invitrogen, USA). The cells were resuspended in Neurobasal™ (Invitrogen) with bioactive supplement B27 (Invitrogen), glutamine (Invitrogen), fetal calf serum (PanEco, Russia) and cultivated under previously developed protocol [8] within 33 days *in vitro* (DIV) on a multielectrode arrays MEA60 (Multichannel Systems, Germany) containing 60 microelectrodes. The arrays were preliminarily sterilized by UV irradiation and treated by polyethylenimine used as reference substrate for cultured cells. Starting density of cell culture was 9000 cells per mm^2. Culture viability was maintained in CO_2- incubator at 35.5°C and gas mixture containing 5% CO_2.

Modeling of hypoxia. Modeling of hypoxia was performed on 33rd day of culture development *in vitro* by replacing the normoxic culture medium by a medium with low oxygen for 10 minutes. The oxygen was displaced from culture medium in sealed chamber where air was replaced by inert gas. When determining the dissolved oxygen concentration using iodometric Winkler titration there was stated that in the aeration of culture medium by inert gas, oxygen concentration decreased from 3.26 (normoxia) till 0.37 ml/l (hypoxic medium).

In the experimental group BDNF (1 ng/ml) was added into culture medium 20 minutes before hypoxia. The control group was composed of the cultures with modeling of hypoxia without preventive application of neurotrophic factor.

Bioelectrical activity registration and analysis. Spontaneous neuronal bioelectrical activity was recorded by means of multielectrode arrays of MEA60 multielectrode system. The arrays consisted of 60 planar round electrodes, each 30 micrometer in diameter, interelectrode distance being 200 micrometer. Extracellular potentials (spikes) characterizing bioelectrical activity of hippocampal culture were recorded in initial state on 33rd day of development *in vitro*, as well as 10, and 120 min later, and every 24 h within 7 days after hypoxia. There were investigated basic characteristics of bioelectrical activity of dissociated hippocampal culture: a number of small network bursts; a number of spikes in a small network burst. The criterion of a small network burst was the presence of spikes on minimum four different electrodes of the arrays, the interspike interval being not above 100 ms.

The obtained data were quantitatively processes and analyzed using MC RACK software of multielectrode system (Multichannel systems, Germany), as well as software package Matlab.

Viability evaluation of cells in dissociated culture. On 3 and 7th day after hypoxia there were calculated the number of cell nuclei stained by propidium iodide (Sigma, USA), and the nuclei stained by bisBenzimide (Sigma). The number of living cells was calculated as percentage ratio between bisBenzimide-positive cells and propidium iodide-positive cells.

Statistical data analysis. The findings were statistically processes using software package Microsoft Excel and Biostat with nonparametric Kruskal–Wallis test. The differences were considered statistically significant in $p<0.05$.

Results. Beginning with the second minute of hypoxia exposure on dissociated hippocampal cultures there was seen spontaneous bioelectrical activity inhibition (Fig. 1). Three minutes after hypoxia by multielectrode arrays in control series none electric events (spike bursts or single spikes) were recorded.

Reoxygenation caused the increase in neuron activity in relation to the initial level. The pattern of bioelectrical activity changed due to the increase of the number of small network bursts (the number of bursts 10 min before hypoxia — 38.20±7.83; after — 101.70±23.67), and insignificant increase in the average number of spikes in a burst (before hypoxia — 251.8±102.1; after — 432.7±141.3). However, 2 h after hypoxia there was irreversible decrease of spontaneous bioelectrical activity (the number of bursts over 10 min — 3.00±5.67; an average number of spikes in a burst — 508.0±232.98).

A day after an acute 10-minute episode of hypoxia there were observed morphological changes in dissociated hippocampal culture as necrotizing cell elements, their number increasing within the following 7 days (Fig. 2). The study of culture viability showed the main part of cells to die on first three days after the exposure. The number of dead cells in the culture increased fourfold compared to normal oxygen conditions, and amounts to 43% of the total cell count.

The study of spontaneous activity pattern has revealed that hypoxic exposure does not only reduce the basic bioelectrical parameters in long-term post-hypoxic period (the number of spikes over 50 ms halves, $p<0.05$) (Fig. 3), but also changes functional characteristics of a network burst that is registered in the form of the change of network burst activation pattern (Fig. 4). Activation pattern is known to be a parameter indicating individual structure of functional neuronal network that changes when exposed to stress factors [9].

The effect of BDNF low concentrations (1 ng/ml) manifested itself in partial preservation of network burst activity during hypoxia, and the normalization of the parameters of spontaneous bioelectrical activity related to the initial level in post-hypoxic period (Fig. 5).

The study of morphological structure and viability of cell culture within 7 days after hypoxia showed the decrease of necrotizing damage and the reduced number of dead

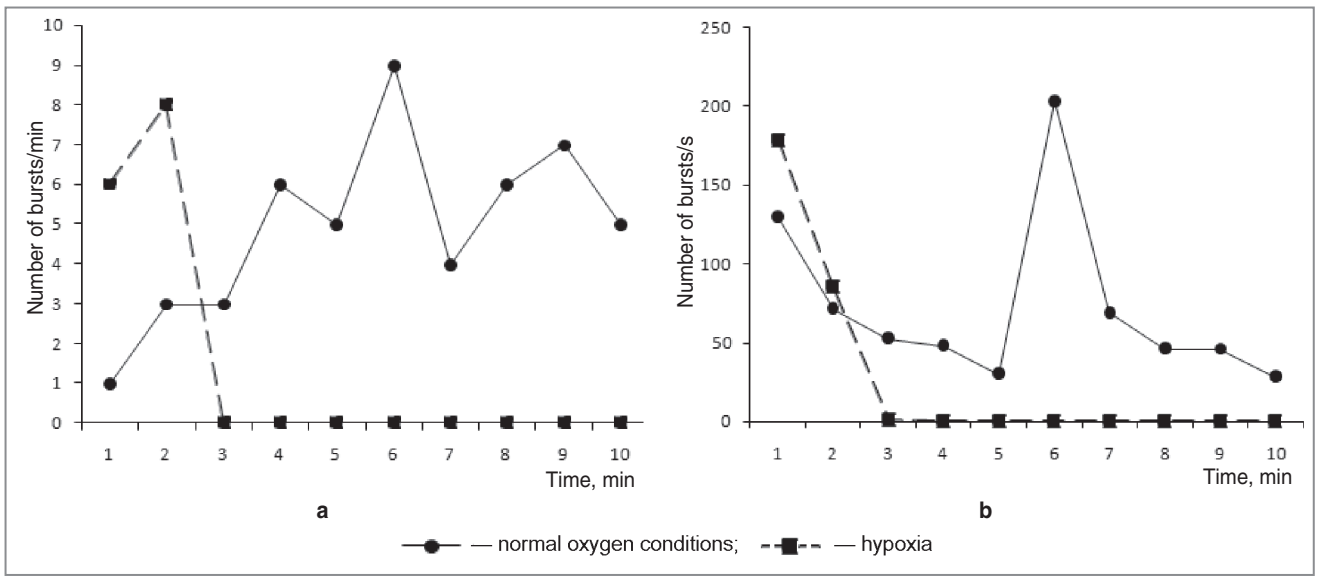

Fig. 1. Spontaneous bioelectrical activity of dissociated hippocampal culture (33 DIV) within 10 min of hypoxia (0.37 ml/l O_2 in culture medium): *a* — the number of small network bursts per minute; *b* — the number of spikes per second

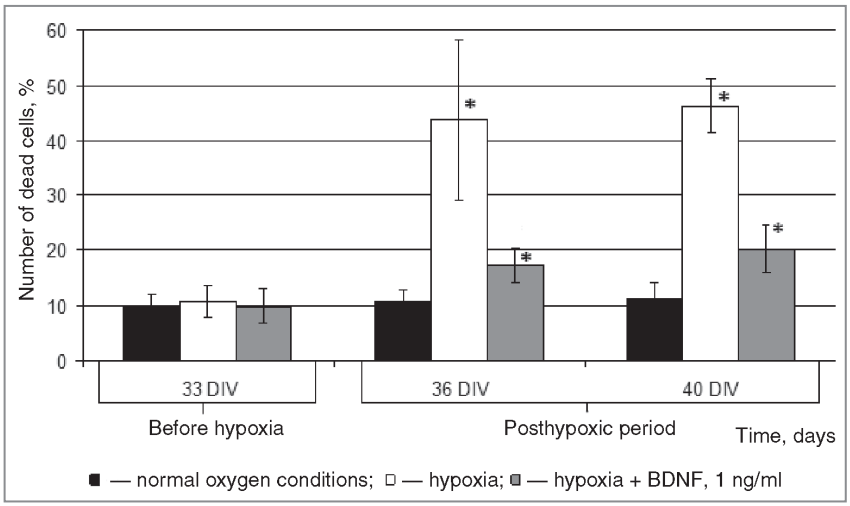

Fig. 2. The number of dead (propidium iodide-positive) cells in dissociated hippocampal culture before hypoxic injury (33 DIV) on 3 (36 DIV), and 7 (40 DIV) day after 10-minute hypoxia (0.37 ml/l O_2); * — statistical significance of differences with initial level ($p<0.05$, Kruskal–Wallis test)

cells in the culture (See Fig. 2) in preventive application of BDNF compared to the control series. 7 days after hypoxia the number of dead cells amounted to 20% of the total cell count in the culture, being 2.35 times statistically significantly lower ($p<0.05$) than in the control series.

Discussion. The carried out experiments revealed 10-minute normobaric hypoxia to cause irreversible changes in spontaneous bioelectrical activity of dissociated hippocampal cultures: the decrease of the number of small network bursts, the number of spikes in a burst, and their total disappearance 2–3 minutes after hypoxia. Moreover, on first three days after hypoxia, the number of dead cells in the culture increases by 4.2 times. The use of BDNF (1 ng/ml) 20 min before hypoxic injury prevents all the revealed negative effects of oxygen deficit. In hypoxic conditions there is partial preservation of bioelectric activity, and after reoxygenation the number of dead cells is statistically significantly ($p<0.05$) lower than in the cultures with no neurotrophic factor treatment.

Anti-hypoxic protective effect of BDNF manifested in the preservation of bioelectrical activity in hypoxia that may be due to the preservation of oxidative phosphorylation as the main function of the mitochondrial cell respiratory system. However, a question of a certain signaling mechanism that is triggered by neurotrophins in hypoxia and results in cell resistance improvement remains unclear. Some authors [3, 10] have shown that neuron viability by means of BDNF is maintained through the activation of extracellular signal-regulated kinase (ERK) or through the pathway of mitogen-activated protein kinases — MAPK-pathway, while other researchers [11–14] have given evidences that in the process there takes part PI-3-kinase signaling mechanism. Moreover, there is the evidence that p38 MAPK (one of signaling pathways) participates in neuroprotection [15, 16]. One of known target molecules MAPK and PI-3-kinase is cAMP-dependent transcription factor (CREB). It is an important component of these signaling pathways leading to gene expression change.

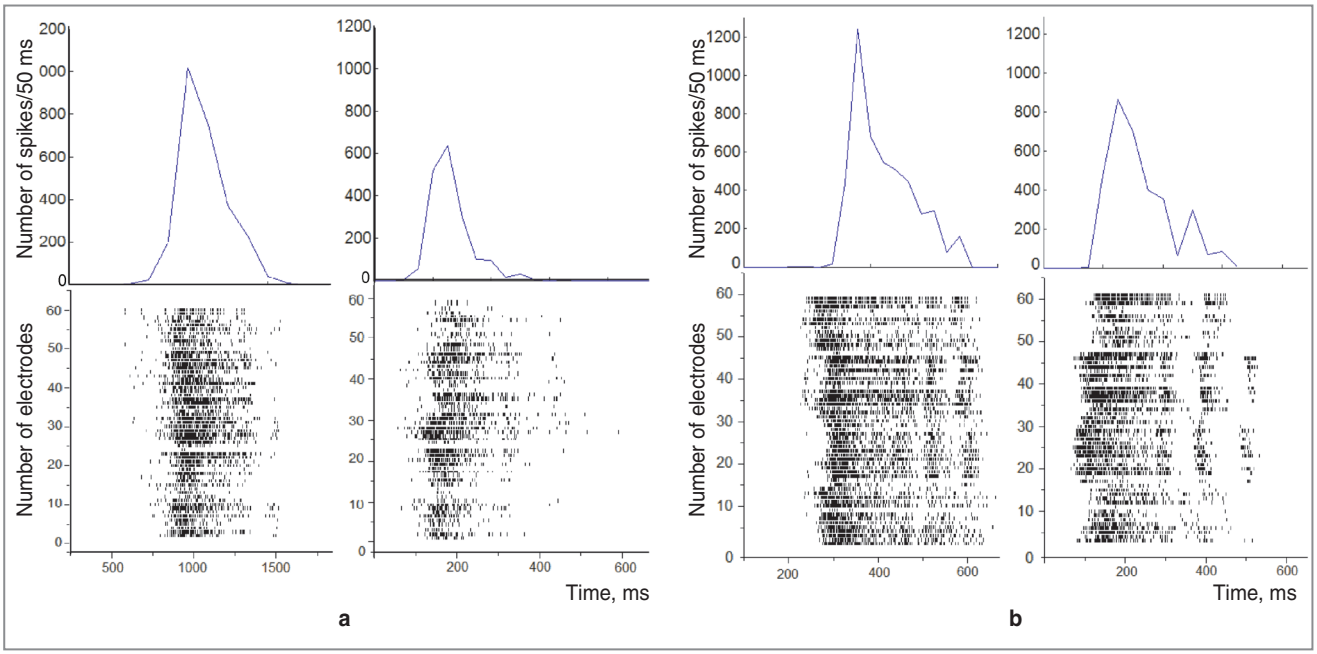

Fig. 3. The number of spikes per 50 ms (at the top) and raster graphs of spontaneous bioelectrical activity of dissociated hippocampal culture (below): *a* — control culture activity before hypoxia (33 DIV) (on the left), and 24 h after the damaging effect (34 DIV) (on the right); *b* — culture activation before BDNF application and hypoxia (33 DIV) (on the left), and 24 h after BDNF application (1 ng/ml) and the damaging effect (34 DIV) (on the right)

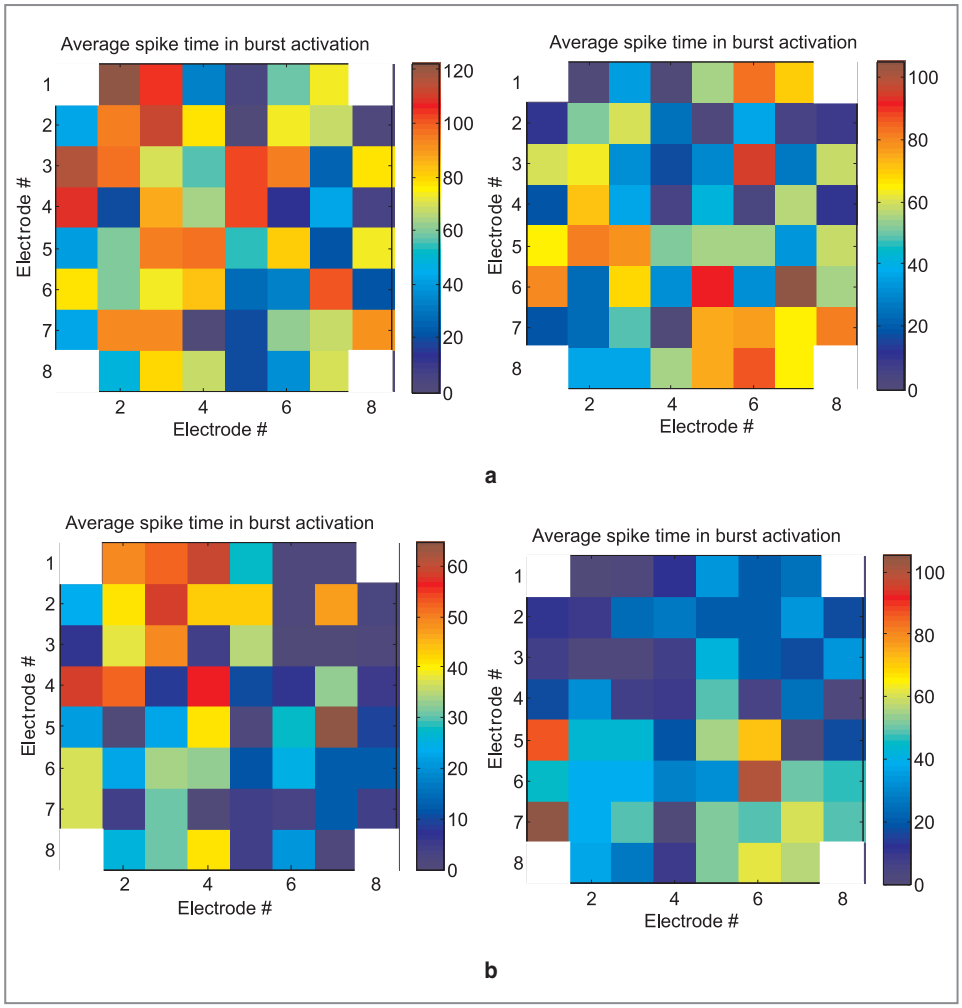

Fig. 4. Pattern of spontaneous bioelectrical activity of dissociated hippocampal culture: *a* — activity of control culture before hypoxia (33 DIV) (on the left), and 24 h after damage effect (34 DIV) (to the right); *b* — culture activity before BDNF application (33 DIV) (on the left), and 24 h after BDNF application (1 ng/ml), and damaging effect (34 DIV) (on the right)

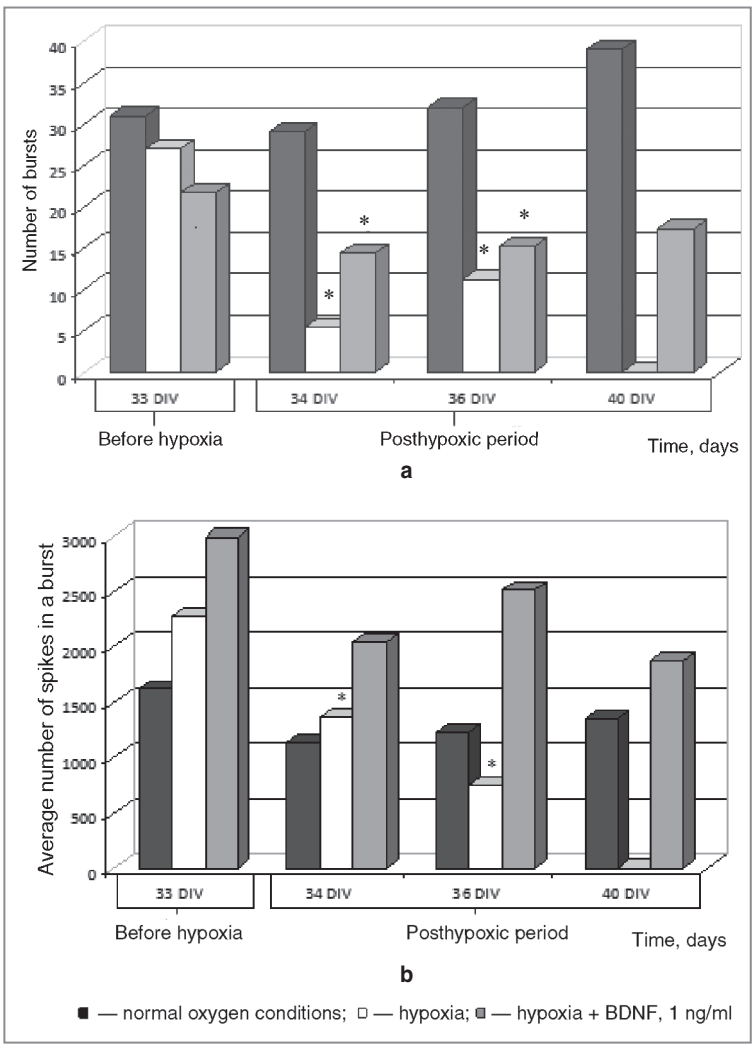

Fig. 5. Spontaneous bioelectrical activity of dissociated hippocampal culture on 1 (34 DIV), 3 (36 DIV), and 7 (40 DIV) day of posthypoxic period: *a* — a number of bursts registered over 10-minute record; *b* — an average number of spikes in a burst per 10 min of registration; * — statistical significance of differences with initial level ($p<0.05$, Kruskal–Wallis test)

CREB is expected to contribute to BDNF-mediated neuron survival in central nervous system activating anti-apoptotic gene expression. Phosphorylation of CREB Ser133 by neurotrophins is required for the activation of CREB-activated transcription factors [17]. Recent *in vitro* study of cortical neurons under circulatory hypoxia (hypoxia/ischemia) [18] has shown exogenic use of BDNF to contribute to the increase of the number of survived cortical neurons after damaging effect through the activation of both MAPK, and PI3-kinase signaling pathways resulting in the activation of CREB phosphorylation. This process to a greater degree is related to extracellular signal-regulated kinase ERK.

In 2012 British researchers have studied *in vitro* the effect of BDNF on oxygen metabolism in mitochondria of murine brain [19]. In case of synaptosome incubation, BDNF effect has been expressed in concentration-dependent increase of respiratory control index (RCI) — the efficiency factor of respiratory chain, ATP synthesis, and organelle integrity. In addition, in the experiments on isolated mitochondria BDNF has not demonstrated a similar effect and has enhanced oxidation only when the substrates of mitochondrial enzymatic complex I have been used. Mitochondrial effect of BDNF has been controlled by MAPK signaling pathway, and rotenone, the inhibitor of mitochondrial enzymatic complex I, *in vitro* and *in vivo* is able to inhibit both mitochondrial, and neuroprotective effect of BDNF.

Conclusion. Preventive BDNF application reduces negative effect of normal barometric hypoxia that enables to consider the neurotrophic factor as the substance having not only neuroprotective but also anti-hypoxic characteristics.

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